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Research Article

First report of *Halomicrobium mukohataei* in Mexico and its biological activity

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Abstract

Haloarchaea produce metabolites of biotechnological importance and grow in hypersaline environments such as coastal lagoons, marine solar salterns, natural brines, and salt lakes. Haloarchaeal compounds from hypersaline environments in Mexico have been scarcely studied. This research aimed to identify a haloarchaea isolate and evaluate the antioxidant, antimicrobial, and cytotoxic properties of the culture's biomass pigments (BPs) and supernatant pigments (SPs). One extremely halophilic archaeal strain designated AS8 was isolated from brine samples of the Bahía de Lobos coastal lagoon, Sonora, and identified as *Halomicrobium mukohataei* based on analyses of the 16S rRNA gene. The SPs showed the best free radical scavenging activity on DPPH and ABTS assays, with 74 and 67% inhibition values, respectively. The extracts also showed significant antibacterial activity against Gram-positive and Gram-negative bacteria with inhibition halos between 7 to 17 mm. Cytotoxic activity of extracts using nauplii of *Artemia salina* showed CL_{50} 850 µg/mL to SPs and > 1000 µg/mL to BPs. This work represents the first isolation study of *H. mukohataei* from Bahía de Lobos lagoon. Likewise, *H. mukohataei* is an alternative source of natural pigments with antioxidant, antimicrobial, and cytotoxic properties, which have potential in biomedical applications and the development of new drugs.

Key words: Haloarchaea, hypersaline environments, pigments

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Introduction

Halomicrobium is a genus of haloarchaea in the family Haloarculaceae, which comprises 16 genera and 224 species (Chaumeil et al. 2020). Haloarchaea make up a group of Archaea that grow in hypersaline environments with NaCl concentrations between 4 and 4.5 M. These microorganisms grow and develop in salt lakes and salt pans and have evolved adaptive mechanisms that allow them to tolerate high NaCl concentrations, low water availability, high temperatures, and ultraviolet radiation (Ventosa et al. 2014; Oren 2015; Bilal-Anwar et al. 2020; Gómez-Villegas et al. 2020).

Haloarchaea are of biotechnological relevance due to their ability to produce enzymes, proteins, carotenoids, exopolysaccharides, polyhydroxyalkanoates, and halocins. Many of these compounds are of biomedical, pharmaceutical, cosmetic, food, industrial, and agricultural interest due to their antioxidant, gelling, and antimicrobial properties (Oren 2010; Singh and Singh 2017; Corral et al. 2020). Recently, the anticancer, antihemolytic, antibacterial, and antioxidant properties of their pigments have been documented (Abbes et al. 2013; Kirti et al. 2014; Yatsunami et al. 2014; Hou and Cui 2018; Giani et al. 2019; Zalazar et al. 2019). Lipophilic bioproducts have been identified in halophilic microorganisms such as *Haloferax mediterranei* and *Natronobacterium gregoryi*. One of the main lipophilic bioproducts is bacterioruberin, a C_{50} acyclic carotenoid with four hydroxyl groups and conjugated double bonds, which increases their scavenging capacity (Lorantfy et al. 2014). Halophilic microorganisms have also been documented producing metabolites with antifungal, antibacterial, and antiviral properties. Some of their identified bioactive compounds include alkaloids, triterpenes, carotenoids, and peptides (Santhaseelan et al. 2022).

Microbial diversity and its biological potential in hypersaline environments have been scarcely studied in Mexico. The objective of this work was the molecular characterization of a haloarchaea isolate from the Bahía de Lobos coastal lagoon, Sonora, Mexico, and the antimicrobial, cytotoxic, and antioxidant evaluation of its pigments.

Material and methods

Brine sample collection

Seventeen brine samples were collected during April 2022 in the Bahía de Lobos located in the Municipality of San Francisco Rio Muerto (27°28'44.2"N, 109°54'44.0"W, 0 m), Sonora, Mexico. The temperature of the brine samples at the time of sampling was 27 ± 2 °C, pH 7.6, and electrical conductivity was > 20 mS/cm. The samples were stored in sterile plastic containers and transported to the laboratory for analysis.

Isolation of the haloarchaea

Each sample was plated on media containing 25% NaCl, following to Rodríguez-Varela et al. (1980), with slight modifications. The modified medium contained, per liter: NaCl 250 g, $MgSO_4 \cdot H_2O$ 20 g, $MgCl_2 \cdot 6H_2O$ 13 g, $CaCl_2$ 1 g, KCl 4 g, NaBr 0.5 g, $NaHCO_3$ 0.2 g, yeast extract 1 g, glucose 10 g, and agar 22 g. After two weeks of incubation at room temperature, pure cultures were obtained by transfer of pigment colonies to agar plates of the medium.

Molecular identification

Genomic DNA was extracted from cells using the method described by Keb-Llanes et al. (2002) with slight modifications. DNA was quantified, and its purity was assessed using a nanodrop spectrophotometer (Thermo Scientific NanoDrop 2000/2000c spectrophotometer). The 16S ribosomal RNA (rRNA) gene was amplified by polymerase chain reaction (PCR) using archaeal-spe-

cific primers: F8 (5'-TTGATCCTGCCGGCCGGAGGCCAT-3') and R1462 (5'-ATCCAGCCGCAGATTCCCCTAC-3') (Lizama et al. 2002). The PCR product was purified using the GENEJET Kit (Thermo Scientific) and sent for sequencing to the DNA Synthesis and Sequencing Unit of the UNAM Institute of Biotechnology (IBT).16S rRNA sequences of haloarchaea were submitted to BioEdit Sequence, Alignment Editor 7.7.1.0, and Unipro Ugene 5.0. Afterward, the sequences were compared with other sequences using BLAST in NCBI GenBank. Phylogenetic analysis was performed using MEGA11 (Tamura et al. 2021).

Inoculum preparation

A young colony of *H. mukohataei* (four days of growth on agar) was added to 10 mL of culture broth containing 25% NaCl. It was then incubated at 37 °C for 7 days to reach a concentration of 1×10^8 CFU/mL, determined spectrophotometrically (turbidity 0.5, λ = 600 nm).

Pigment production

Five mL of the inoculum were added to 500 mL of liquid medium in 1 L Erlenmeyer flasks. The flasks were incubated at room temperature for 10 days in a rotary shaker at 150 rpm (Fig. 1).

Pigment extraction

Liquid cultures were centrifuged at 10 000 xg for 25 min at room temperature to separate the cell biomass from the culture medium. Two milliliters of deionized water were added to the cell pellet and sonicated for 1 minute to promote cell lysis. Acetone was then added and kept in agitation at 150 rpm for 12 h, protected from light, filtered, and concentrated at a rotary evaporator to generate the biomass pigments (BPs). While the bioactive compounds excreted in the remaining liquid medium were recovered by liquid-liquid extraction of 500 mL of culture medium with acetone, the organic phase was concentrated in a rotary evaporator at 50 °C to generate the supernatant pigments (SPs) (Fig. 1).

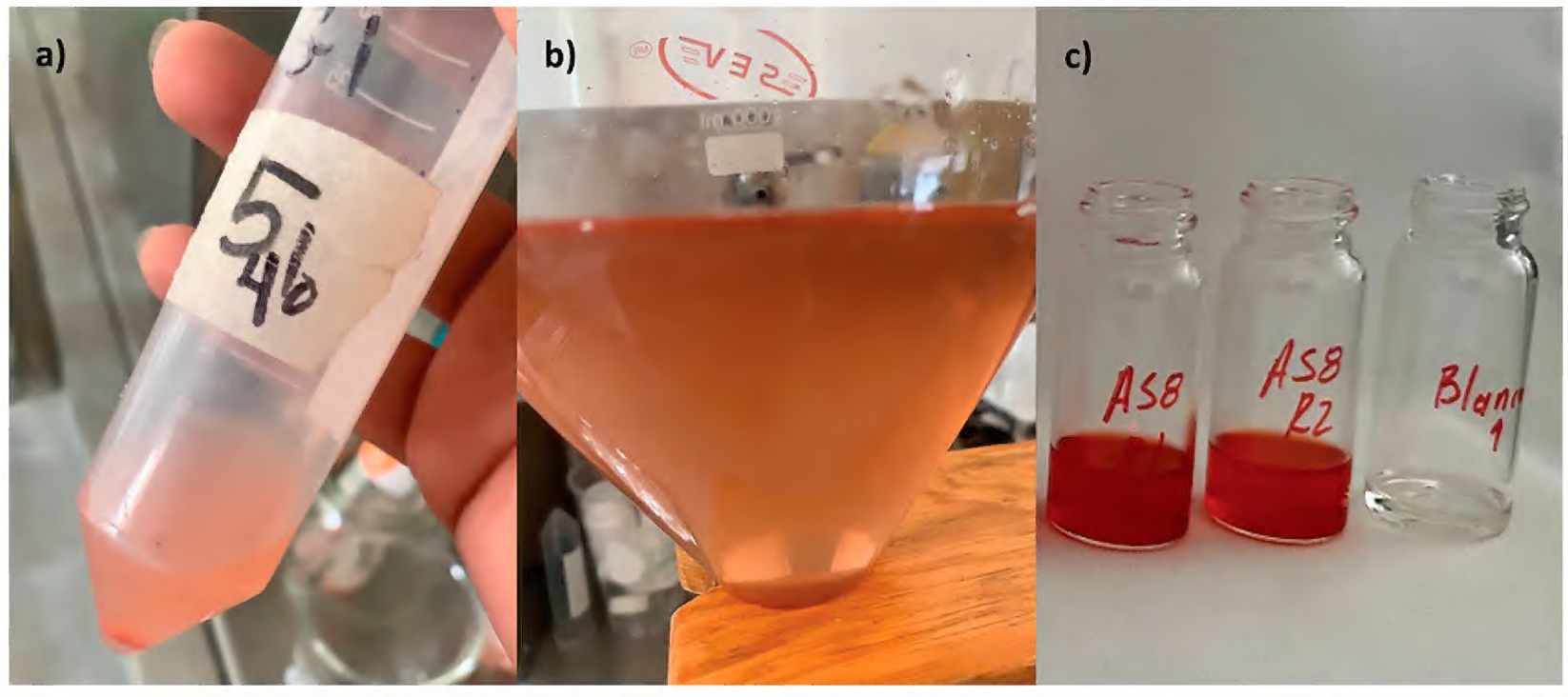

Figure 1. Pigment production from haloarchaea isolate from Bahía de Lobos **a** extraction of biomass pigments (BPs) **b** liquid-liquid extraction of the supernatant pigments (SPs) **c** color of the pigments after concentration in the rotary evaporator.

Antioxidant activity

DPPH and ABTS assays

The pigment solutions were prepared at different concentrations: 5, 2.5, 1.2, 0.6, 0.3, and 0.15 µg/mL (Moraes-de-Souza et al. 2008). The antioxidant activity was determined by the methods of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay and the 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) disodium salt radical cation ($ABTS^{\cdot+}$). A solution of DPPH 60 µM in methanol was prepared. The absorbance values at 517 nm were recorded after mixing 975 µL of DPPH solution and 25 µL of each pigment solution (A_{sample}). $A_{control}$ was the absorbance of the mixture of 900 µL of DPPH solution and 100 µL of methanol. The ABTS assay was performed according to the method of Re et al. (1999), which was slightly modified. The $ABTS^{+}$ radical cation was generated by the reaction of ABTS and potassium persulfate in an aqueous solution for 16 h at room temperature protected from light. Briefly, 25 µL of each pigment concentration was added to 975 µL of ABTS solution. Afterward, it was incubated at room temperature for 6 minutes and then read at an absorbance of 734 nm. The scavenging activity was calculated: radical scavenging activity (%) = $[(A_{control}-A_{sample})/A_{control}]\times100$. β-Carotene was used as the control. All assays were performed in triplicate.

Antimicrobial activity

The antimicrobial activity of pigments was evaluated by disk diffusion assay against seven strains pathogenic to humans: *Bacillus* sp., *Candida albicans*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. According to McFarland's standard, all strain inoculums were adjusted to a turbidity of 0.5. Sterile 6 mm filter paper discs were placed on the agar plates and impregnated with 6 µL of pigments dissolved in ethanol at a concentration of 2 mg/mL. The plates were incubated for 24 h at 37 °C. Inhibition halos were measured to evaluate antimicrobial activity. Cefotaxime and fluconazole were positive controls for bacteria and *C. albicans*, respectively. Discs impregnated with ethanol were used as a negative control. Experiments were performed in triplicate (Balouiri et al. 2016).

Cytotoxic assay

The cytotoxicity of archaeal pigments was determined by a lethality bioassay using the protocol of Meyer et al. (1982). *Artemia salina* cysts were incubated in synthetic seawater for 24 h at room temperature and with a light source. Pigment solutions were prepared at 1, 0.1, and 0.01 mg/mL concentrations in 1.5% DMSO. Then, in 96-well microplates, 200 µL of each pigment solution was added, followed by 15 to 20 nauplii per well, and incubated for 24 h at room temperature. A 1.5% DMSO solution was used as a blank. The percentage of dead nauplii was determined. The LC_{50} was determined by interpolation with GraphPad Prism 5.0 software.

Preliminary characterization of the pigments

Pigment analysis by thin-layer chromatography (TLC)

TLC analysis was performed as described by Hou and Cui (2018). The pigments were spotted onto a TLC plate (silica gel 60 F254, Merck, Germany) and developed in 50% (v/v) acetone in n-heptane.

Pigment analysis by Fourier-transform Infrared (FT-IR) spectroscopy

FT-IR spectroscopy is a fast method that shows the absorption of infrared light by molecular bonds at a given wavelength. This absorption is associated with molecular vibrations. Thus, an FT-IR spectrum can be divided into regions showing the characteristic IR bands of specific functional groups.

Approximately 1 mg of the pigments and 0.5 mg of the analytical standard β-carotene were analyzed by FT-IR. Spectral data were obtained on an FT-IR spectrometer (Nicolet 6700, Thermo Scientific). The pigments' FT-IR spectra were recorded in transmittance mode within the wavenumber range 4000-750 cm^{-1} with 128 scans. The spectra were collected and processed using OMNIC spectra (Thermo Scientific).

Statistical analysis

The results were expressed as the mean ± SD. The data were submitted to one-way variance analysis (ANOVA). Using GraphPad Prism 5.0 software. All experiments were performed in triplicate.

Results

Isolation of haloarchaea

A total of 96 haloarchaea isolates were obtained from brine samples from Bahía de Lobos on medium agar supplemented with 25% NaCl. Colonies with red coloration were selected for study. The BPs and SPs were obtained with a yield of 28 mg g^{-1} of biomass and 140 mg L^{-1} of supernatant. The strain isolated from the coastal lagoon Bahía de Lobos is a reddish strain, circular in shape, slightly mucoid, with colony sizes ranging from 0.5 to 2 mm, tolerates 25% NaCl, and is Gram-negative (Fig. 1).

Molecular characterization of haloarchaea

To identify strains, 16S sequences isolated were compared with other sequences using BLAST available in GenBank. The maximum likelihood method and the Tamura-Nei + G + I model were used to establish the evolutionary history. The sequences used were obtained from NCBI; *Halorubrum saccharovorum* (NR_119144), *Halorubrum lacusprofundi* (NR_028244.1), *Haloferax denitrificans* (NR_028215.1), *Haloferax gibbonsii* (NR_028213.1), *Haloferax mediterranei* (NR_028212.1), *Halococcus morrhua* (X00662.1), *Halococcus dombrowskii* (NR_113431.1), *Halobacterium salinarum* (NR_113057.1), *Halobacterium halobium* (M11583.1), *Haloarcula*

marismortui (X61688.1), *Haloarcula vallismortis* (D50851.1), *Halomicrobium mukohataei* (LT634699.1), and *Thermococcus celer* (NR_042736.1). The percentage of trees in which the associated taxa were grouped is shown next to the branches. The tree is drawn to scale, with branch lengths measured in several substitutions per site. This analysis involved 14 nucleotide sequences with 1497 positions in the final data set. Phylogenetic analysis exhibited that isolate AS8 was *Halomicrobium mukohataei* with an E value of 0,0 with respect to the sequence registered with accession number PV290302 (Fig. 2).

Antioxidant activity of pigments

Both pigments extracted from the isolates increased their scavenging effect compared to the control. The pigments showed concentration-dependent antioxidant activity in the DPPH and ABTS assays. The SPs showed better antiradical activity than the BPs and β-carotene in both methods, with 74, 67, and 38%,

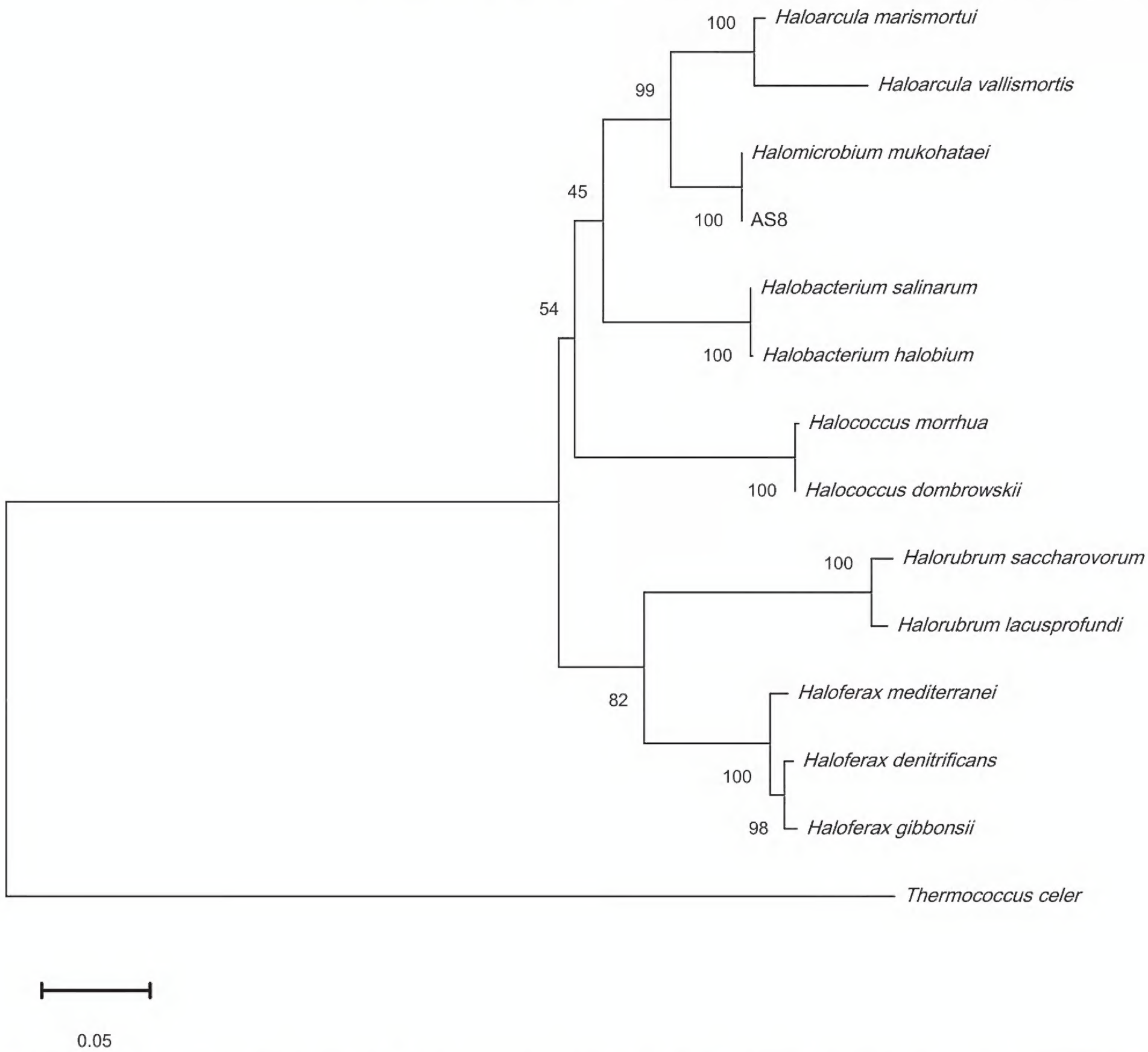

Figure 2. Phylogenetic tree based on 16S rRNA gene sequences showing the position of haloarchaea isolate AS8 from Bahía de Lobos coastal lagoon, Sonora, Mexico. Scale bar represent substitutions per nucleotide site.

respectively, by the DPPH method. By the ABTS method, the antioxidant activity was 67, 52, and 34%, respectively (Fig. 3). Pigments produced by haloarchaea exhibited important antioxidant compounds that could help human health and biotechnological applications.

Antimicrobial activity of pigments

The BPs and SPs exhibited antibacterial activity against *Bacillus* sp., *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. *Bacillus* sp showed the highest sensitivity to the pigments tested, followed

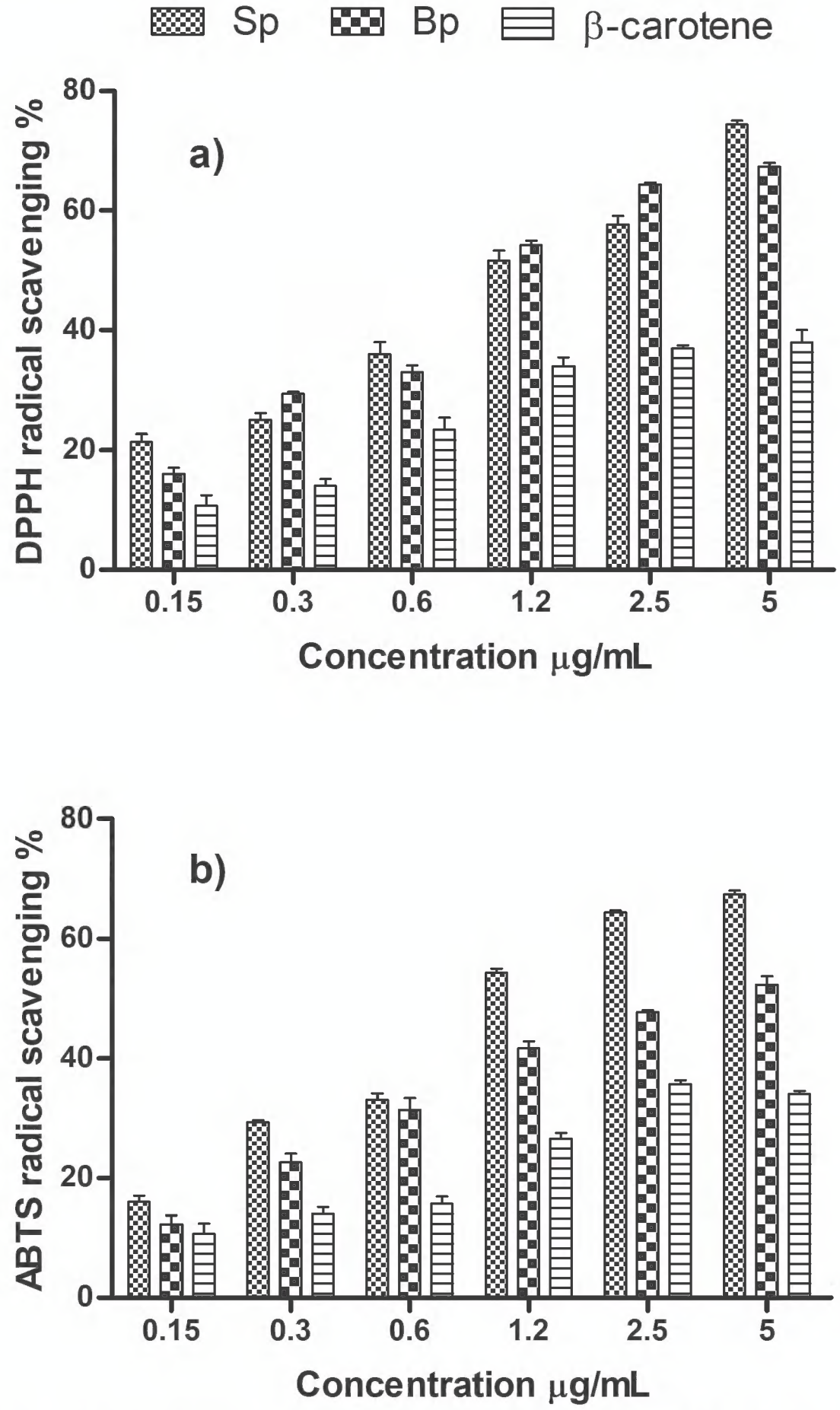

Figure 3. Radical scavenging activity of pigments from haloarchaea isolate AS8 (*Halomicrobium mukohataei*) from Bahía de Lobos coastal lagoon, Sonora, Mexico **a** 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay **b** 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) disodium salt radical cation (ABTS•+). Data are represented as the mean of three replicates.

by *S. aureus* and *K. pneumoniae*. The pigments did not display antifungal activity against *Candida albicans* (Table 1). The SPs showed a better antibacterial effect than BPs. The data revealed that Gram-positive bacteria are more sensitive to pigments than Gram-negative bacteria. While the pigments had no anti-*Candida albicans* effect, these results are significant and provide new avenues for future investigations of antibacterial molecules from haloarchaea.

Cytotoxic activity of pigments

The BPs showed a 40% mortality rate of *A. salina* nauplii at the 0.1 and 1 mg/mL concentrations. In comparison, the SPs showed 45% lethality of nauplii at the 1 mg/mL concentration and 40% at the 0.1 mg/mL concentration.

Pigment analysis by thin-layer chromatography (TLC)

Pigment analysis by TLC showed a similar chromatographic profile in both pigments. However, the SPs (Line 4) show a higher intensity in the red-pink signal with Rf = 0.45 than the BPs, suggesting a higher concentration of these components in the SPs. The components observed in spot 1 with Rf = 0.45 (Fig. 4) indicate the presence of carotenoid-like components such as bacterioruberin.

FT-IR analysis of pigments

FT-IR spectra were analyzed to find the chemical structures of carotenoids in the pigments; the results are shown in Fig. 5. The FT-IR analysis revealed the presence of several peaks at 1724, 1600, 1580, 1461, 1380, 1267, 1119, 1070, 1039, 950, 741 and 704 cm^{-1} for the SPs (Fig. 5A) and the BPs (Fig. 5B). Most of the signals were shared with the β-carotene standard (Fig. 5C). This suggests that the pigments contain carotenoid-like components. The band at 1461 cm^{-1} corresponds to the vibration of methylene groups, and the peak at 1380 cm^{-1} is attributed to vibrations of CH bonds. Interestingly, the 1267 cm^{-1} band corresponding to COH vibrations appears in the studied pigments. These characteristic signals could confirm the presence of bacterioruberin (Fig. 5C).

Table 1. Antimicrobial activity of the pigments extracted from haloarchaea isolate AS8 (*Halomicrobium mukohataei*).

Microorganism	Inhibition zones (mm)		
	BPs	SPs	Positive control
Gram (+)			
Bacillus sp	13 ± 1	17 ± 0.5	23 ± 1
Enterococcus faecalis	7 ± 0.5	9 ± 1	18 ± 1
Staphylococcus aureus	11 ± 0.5	12 ± 1	20 ± 2
Gram (-)			
Escherichia coli	9 ± 1	9 ± 0.5	18 ± 2
Klebsiella pneumoniae	10 ± 1	11 ± 1	19 ± 1
Pseudomonas aeruginosa	9 ± 0.5	7 ± 0.5	19 ± 2
Yeast			
Candida albicans	0	0	20 ± 2

BPs, biomass pigments; SPs, supernatant pigments. Results expressed as mean ± standard deviation.

Figure 4. Analyses of the pigments of haloarchaea isolate AS8 (*Halomicrobium mukohataei*) by thin layer chromatography (TLC). Line 4: supernatant pigments (SPs), Line 6: biomass pigments (BPs).

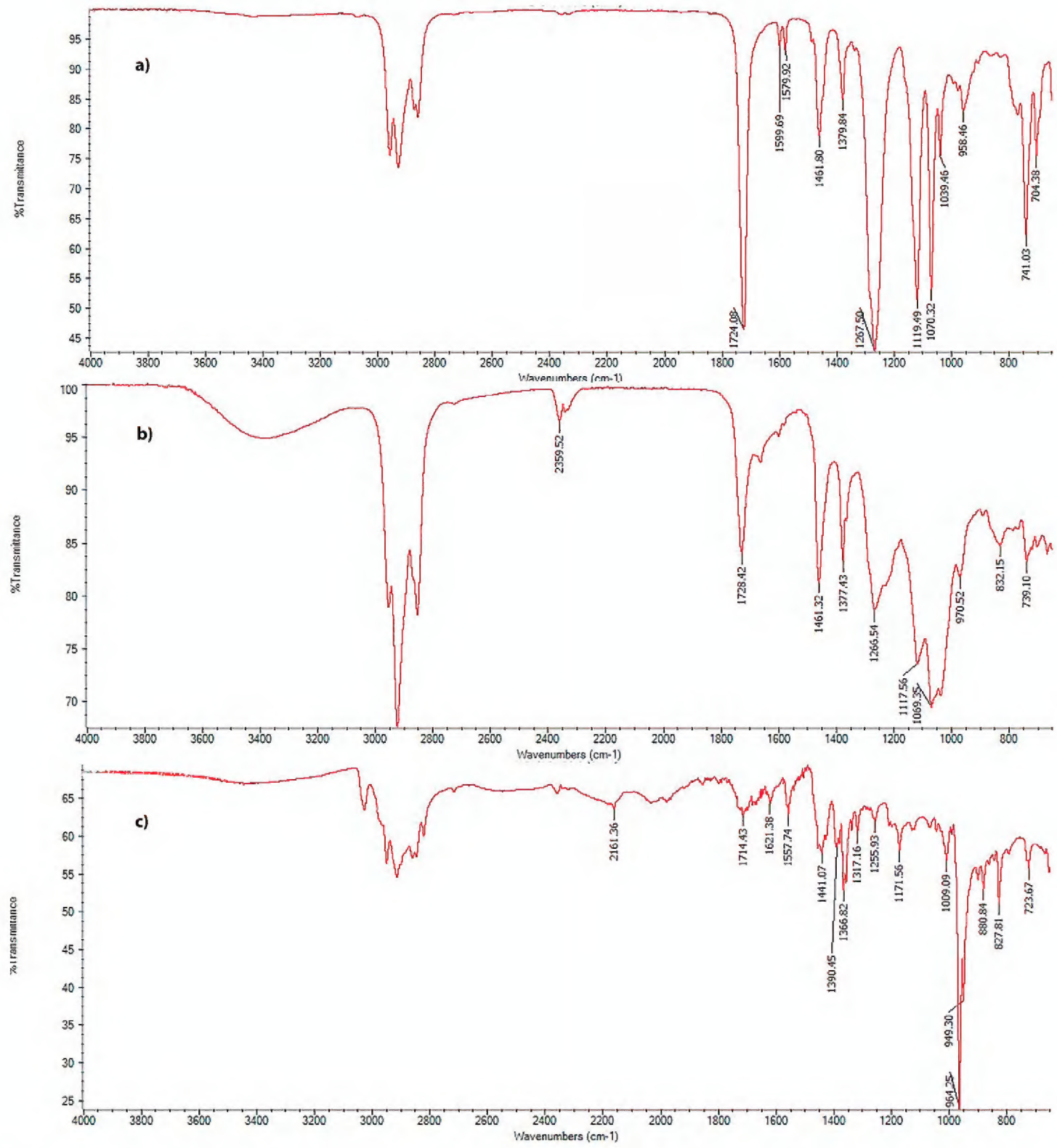

Figure 5. FT-IR chromatograms of pigments extracted from haloarchaea isolate AS8 (*Halomicrobium mukohataei*) **a** supernatant pigments (SPs) **b** biomass pigments (BPs) **c** β-Carotene.

Discussion

The ability to make physiological changes in environments with different stress types forces microorganisms to adapt to extreme conditions by developing specific responses to stress (Oren 2014; Oren 2015). One such response is the gen-

eration of pigments and exopolysaccharides by haloarchaea in response to salt stress (Squillaci et al. 2015). These pigments are of biotechnological relevance (Kaur and Singh 2011; Giani et al. 2019; Giani et al. 2021). The production of carotenoids by various halophilic microorganisms has been documented (Abbes et al. 2013; Hou and Cui 2018; Hegazy et al. 2020). Likewise, bacterioruberin, lycopene, and carotenes from *Halorubrum* pigments have been reported (Sahli et al. 2020).

16S rRNA gene sequencing has revolutionized prokaryote taxonomy and identification, positioning itself as a powerful tool (Rodicio and Mendoza 2004). The evolutionary stability of the 16S rRNA gene, its universal presence, and ease of amplification make it ideal for phylogenetic analysis from domain to genus level. However, it has limitations at the species level (Stackebrandt 2009). While 16S rRNA gene sequencing remains the gold standard, other genes like rpoB differentiate closely related species (Christensen and Olsen 2018). However, our research had no conflict in distinguishing between species using only the 16S rRNA gene.

Molecular identification of strain AS8 based on analyses of the 16S rRNA gene found 100% similarity with *Halomicrobium mukohataei*, initially described as *Haloarcula mukohataei* and isolated for the first time from salt pans in Argentina by Ihara et al. (1997) However, morphology, polar lipid composition, and further phylogenetic studies have provided information to determine that this microorganism does not belong to the genus *Haloarcula* (Oren et al. 2002). This study corresponds to the first report in Mexico of the presence of this species.

Many microorganisms produce pigments with antimicrobial, antioxidant, and cytotoxic properties (Giani et al. 2019). Prodigiosin pigment produced by *S. marcescens* exhibits antibacterial and antitumor properties (Lapenda et al. 2015). Similarly, in our study, the pigments produced by *H. mukohataei* show antibacterial activity against human pathogenic microorganisms, including *Bacillus* sp., *E. faecalis*, *P. aeruginosa*, *K. pneumoniae*, and *E. coli*. However, they did not show antifungal activity against *C. albicans*. The aforementioned results are also comparable with those reported by Gómez-Villegas et al. (2020), who reported significant antimicrobial activity of pigments from *Haloarcula hispanica* and *Halorubrum salinarum*, against three Gram-positive and one Gram-negative pathogenic bacteria: *Bacillus cereus*, *Micrococcus luteus*, *Staphylococcus aureus*, and *Escherichia coli*. Sahli et al. (2020) reported the antimicrobial effect of pigments of *Halorubrum* sp against *S. aureus*, *K. pneumoniae*, *P. aeruginosa*, and *E. coli*.

Several studies have reported the antioxidant activity of halophilic microorganisms (Ying-Chao et al. 2023; Giani et al. 2022; Flores et al. 2020). Gómez-Villegas et al. (2020) reported that *H. hispanica* and *H. salinarum* pigments showed 90% and 66% radical scavenging activity. Similarly, in this work, the pigments evaluated showed radical scavenging activity between 68% and 75%. A higher concentration of carotenoid-type components in the SPs could explain such effectiveness concerning BPs. The antioxidant capacity can be attributed to carotenoid-type components. Pigments extracted from the isolate showed higher antioxidant capacity than the standard antioxidant β-carotene. Similar results were found with *Halopelagius inordinatus*, *Halogranum rubrum* and *Haloferax volcanii*. The antioxidant capacities of pigments produced by these strains were higher than the standard β-carotene (Hou and Cui 2018).

The pigments´ cytotoxic activity on *A. salina* nauplii was categorized according to the reports by Fernández-Calienes et al. (2009), which consider that extracts with CL_{50} < 10 mg/mL are highly toxic, < 100 mg/mL are mod-

erately toxic, and > 1000 mg/mL are non-toxic. According to this classification, the BPs and SPs from the AS8 isolate are moderately toxic. This bioassay provides information regarding a first screening of the cytotoxic activity that they may exert on cancer cell lines.

Spot 1 observed in the TLC analysis of the pigments exhibits the same Rf and color value reported for bacterioruberin (Hou and Cui 2018). Similarly, FT-IR analysis of the pigments shows binding vibrations for COH, confirming that one of the pigment components is bacterioruberin. The better biological activity observed for SPs may be due to a higher concentration of bacterioruberin in those pigments, which could explain the higher intensity of the chromatographic signal for SPs (Spot 1, Line 4) than for BPs, and the similar intensities of the signals in the FT-IR chromatograms.

Conclusion

This study is the first investigation of the antimicrobial, antioxidant, and cytotoxic activity of pigments produced by *Halomicrobium mukohataei* and the first report of the presence of this species in the coastal lagoon Bahía de Lobos in Sonora, Mexico. In addition, these pigments' bioactive potential shows their biomedical relevance. Bacterioruberin is one of the main components of the pigments produced by *H. mukohataei*. This makes it an alternative natural pigment with antimicrobial, antioxidant, and cytotoxic properties, with potential in biomedical applications and new drug development.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: JLHM, SPC, DCL. Data curation: SPC. Formal analysis: DCL, JLHM. Investigation: EGS. Software: JLHM. Validation: DCL. Writing - original draft: EGS, DCL. Writing - review and editing: JLHM, SPC, TAB, EGS.

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Data availability

All of the data that support the findings of this study are available in the main text.

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